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Ctp1^{CtIP} and the Rad32^{Mre11} nuclease activity are required for Rec12^{Spo11} removal but Rec12^{Spo11} removal is dispensable for other MRN-dependent meiotic functions

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Abstract

The evolutionarily conserved Mre11/Rad50/Nbs1 (MRN) complex is involved in various aspects of meiosis. Whereas available evidence suggests that the Mre11 nuclease activity might be responsible for Spo11 removal in *Saccharomyces cerevisiae*, this has not been experimentally confirmed. This study demonstrates for the first time that Mre11 (*Schizosaccharomyces pombe* $Rad32^{Mre11}$) nuclease activity is required for the removal of $Rec12^{Spo11}$. Furthermore, we show that the CtIP homologue Ctp1 is required for $Rec12^{Spo11}$ removal, confirming functional conservation between $Ctp1^{CtIP}$ and the more distantly related Sae2 protein from *S. cerevisae*. Finally, we show that the MRN complex is required for meiotic recombination, chromatin remodelling at the *ade6-M26* recombination hot spot, and formation of linear elements (which are the equivalent of the synaptonemal complex found in other eukaryotes), but that all these functions are proficient in a *rad50S* mutant, which is deficient for $Rec12^{Spo11}$ removal. These observations suggest that the conserved role of the MRN complex in these meiotic functions is independent of $Rec12^{Spo11}$ removal.

Introduction

In meiosis, one round of DNA replication is followed by two nuclear divisions that divide the genetic material equally over four haploid daughter cells. Meiotic recombination contributes to genetic diversity and is essential for correct disjunction of the homologous chromosomes in the first meiotic division. In meiotic prophase, after meiosis-specific DNA replication, the homologous chromosomes pair and recombine. In the following two nuclear divisions the homologous chromosomes (meiosis I) and the sister chromatids (meiosis II) are segregated. The study of meiosis in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has greatly contributed to our understanding of various meiotic processes. As these model organisms are as distantly related to each other as to animals (35), detailed studies of similarities and differences between meiotic mechanisms in these yeasts are informative as to which mechanisms are conserved in higher eukaryotes.

The evolutionarily conserved Mre11/Rad50/Nbs1 (MRN) protein complex is involved in a wide range of early responses to DNA damage. Mutations in Nbs1 and Mre11 are responsible for the cancer prone human disorders Nijmegen Breakage Syndrome and Ataxia Telangiectasia-Like Disorder. Central in this complex is the Mre11 nuclease, which is thought to be involved in DSB end resection and DSB signalling (reviewed in 40).

The MRN complex is also involved in multiple aspects of meiosis. In *S. cerevisiae*, meiotic recombination is initiated by the topoisomerase-like protein Spo11 (17), which creates a double strand break (DSB) in the DNA. Spo11 remains covalently

bound to the 5' end of the break ends (17) and is removed by endonucleolytic cleavage (28) to initiate subsequent DSB end resection and meiotic recombination. Meiotic DSB formation is abolished in *S. cerevisiae* MRN null mutants (6, 16). In an *S. cerevisiae rad50S* point mutant (a separation of function mutant with severe defects in meiosis, but only mild defects in mitotic DNA repair; 2) meiotic DSBs are formed, but this mutant is unable to remove Spo11 from meiotic DSB ends (17). This observation has implicated the MRN complex in Spo11 removal. As a nuclease dead *mre11-D56N* mutant is defective in resecting meiotic DSBs (27), it has been proposed that the Mre11 nuclease activity is responsible for Spo11 removal, but this has not been experimentally confirmed. Also in *S. pombe*, meiotic DSBs are formed by the Spo11 homologue, called Rec12 (7). An *S. pombe rad50S* mutant is defective in DSB repair and this feature has been instrumental in the study of DSB formation in this organism (42). Although this phenotype is compatible with an involvement of the MRN complex in $Rec12^{Spo11}$ removal, this has not been demonstrated experimentally.

Meiotic DSB formation in *S. cerevisiae* (30) is accompanied by an increase of micrococcal nuclease (MNase) sensitivity, suggesting that a more open chromatin structure could facilitate DSB repair. *S. cerevisiae* Mre11 is required for meiosis specific chromatin remodelling, whereas Rad50 and Xrs2 (the *S. cerevisiae* Nbs1 homologue) are dispensible (29). Also in *S. pombe*, meiotic recombination hot spot activity at *ade6-M26* has been associated with increased MNase sensitivity (23), but a role of MRN in this process has not been reported.

In the great majority of sexually reproducing eukaryotes a meiosis-specific

63 tripartite structure is formed during meiotic prophase: the synaptonemal complex (SC),
64 which is thought to be involved in various processes associated with chromosome pairing
65 and recombination. Early in meiotic prophase, axial elements are formed along the sister
66 chromatids of the individual chromosomes. Pairing and connection of the axial elements
67 by transverse filaments leads to formation of the tripartite SC (in which the axial
68 elements are now called lateral elements; 31). In *S. cerevisiae rad50Δ* and *rad50S*
69 mutants SC precursors are formed, but formation of a complete SC is blocked (2). In *S.*
70 *pombe* WT cells, no fully formed SC is found, but instead linear elements (LEs) appear
71 during meiotic prophase that show similarity to SC precursors in other organisms (4). It
72 remains unknown if the MRN complex is involved in LE formation in *S. pombe*.
73 Mutants of *sae2* in *S. cerevisiae* have a *rad50S*-like phenotype, in meiosis as well
74 as in mitotic cells (32), and it has been shown that Sae2 is required for Spo11 removal in
75 meiosis (28). Recently, a novel gene, called *ctp1*, was identified in *S. pombe* (21,
76 1) which shows homology to the mammalian tumour suppressor CtIP (33) and the more
77 distantly related Sae2 in *S. cerevisiae.* CtIP/Ctp1 has been shown to interact with the
78 MRN complex (33) and is involved in DSB end resection (21, 33), but a role for Ctp1 in
79 $Rec12^{Spo11}$ removal in *S. pombe* has not been confirmed.
80 Whereas meiotic phenotypes of MRN and *sae2* mutants have been extensively
81 studied in *S. cerevisiae*, much remains unknown about the role of the MRN complex and
82 $Ctp1^{CtIP}$ in *S. pombe* meiosis. In this study, we characterise meiotic phenotypes of *S.*
83 *pombe rad50* and $rad32^{mre11}$ null and separation of function mutants. First we
84 demonstrate, for the first time in any organism, that the nuclease activity of $Rad32^{Mre11}$ is

89 required for Rec12^{Spo11} removal. Second, we demonstrate that Ctp1^{CtIP} is required for
90 Rec12^{Spo11} removal, confirming functional conservation between the distantly related *S.*
91 *pombe* Ctp1^{CtIP} and *S. cerevisiae* Sae2 proteins. Finally, we show that *S. pombe rad50S*
92 has a defect in removing Rec12^{Spo11}, but is proficient for meiotic recombination
93 (measured in surviving spores), chromatin remodelling at the *ade6-M26* recombination
94 hot spot, and LE formation, whereas all of these functions are defective in *rad50Δ*.

Materials and methods

Yeast strains and techniques

For strain construction and propagation standard genetic methods and media were used (11). Strains used and constructed in this study are listed in Table 1.

Previously published procedures

Measurement of meiotic spore viability and recombination (11), synchronisation of meiotic cultures (4, 7), pulsed field gel analysis (7), preparation of chromosome spreads and electron microscopy (4) and analysis of meiotic nucleosome remodelling at *ade6-M26* (23) were described previously.

DNA-linked protein detection assay

We developed this assay based on previously published procedures (17, 34). Pre-meiotic or meiotic cells (25 ml) were washed in 1 ml lysis buffer (8 M Guanidine HCl; 30 mM Tris, 10 mM EDTA, 1 % Sarcosyl, pH 7.5), resuspended in 750 µl lysis buffer and lysed using glass beads (± 0.8 g). The cell extract was incubated at 70 °C for 15 minutes: these strongly denaturing conditions remove non-covalently bound proteins from the DNA. After clarification (15' 13.000 RPM in an eppendorf centrifuge), one aliquot of extract was set aside for DNA quantification (see below) while the rest was loaded on a CsCl gradient, consisting of 1 ml layers with densities of 1.82, 1.72, 1.50 and 1.45 $^{g}/_{ml}$ respectively. The gradients where centrifuged for 24 hours at 30.000 RPM in a Sorvall AH650 rotor to separate the free proteins from the DNA.

To ensure equal DNA loading, the DNA concentration in the extract was measured and this value was used to adjust the volume of the fractions loaded on the slot blot. For this purpose, the aliquots of extract which were set aside for DNA quantification were treated overnight with RNase (0.5 $^{\mu g}/_{ml}$) and the DNA concentration was determined fluorimetrically using PicoGreen (Molecular Probes/Invitrogen detection technologies). After centrifugation, the gradients were fractionated into 0.5 ml fractions and adjusted amounts were loaded onto a slot blot.

To detect the presence of covalently bound HA-tagged $Rec12^{Spo11}$ in the DNA fractions the membrane was probed with a monoclonal antibody (Santa Cruz sc-7392). The membrane was processed using standard Western blot procedures and visualised using chemiluminescence. Using this procedure, control cultures of untagged strains only showed slight cross hybridisation with the top 2 fractions (9 and 10) from the CsCl gradient, which contain the free proteins. These fractions do not contain any DNA, are difficult to load on a slot blot as they tend to clog the membrane, and are therefore not loaded for most experiments. Slot blots of pre-meiotic cells showed no $Rec12^{Spo11}$ signal in any of the DNA containing fractions (data not shown).

Results

S. pombe rad50S is temperature sensitive for meiotic spore viability and DSB repair and deficient for Rec12^{Spo11} removal

We have previously created a *rad50S* mutant (*rad50-K81I*) which has been instrumental in the study of meiotic DSB formation in *S. pombe* (42). As previously reported (10), we find that *rad50S* is temperature sensitive for meiotic spore viability (Fig. 1a). At 34 °C the spore viability is 0.6 % (similar to that of *rad50Δ*). At 25 °C it is 14.6 %. As has been previously shown for *rad50Δ* (41), the low spore viability of *rad50S* at 34 °C is rescued by deletion of *rec12^{spo11}*, suggesting that the reduced viability is due to a DSB repair defect in this mutant. To test if the temperature sensitive spore viability phenotype of *rad50S* indeed reflects a defect in DSB repair, we looked at meiotic DSB formation and repair in a meiotic time course of *rad50S* at permissive and restrictive temperature using Pulsed Field Gel Electrophoresis (PFGE; Fig 1b). We found that at 34 °C the intact chromosomes are transformed into broken DNA fragments that remain unrepaired. At 25 °C most DNA gets transformed into broken fragments, but a significant fraction of chromosomal DNA is intact near the end of the timecourse. After approximately 8 hours the intensity of the intact chromosomal DNA bands starts to diminish. At this time, the majority of the cells have sporulated (see Fig. 1c); spore wall formation makes the spores resistant to lysis preventing the DNA to enter the gel. We conclude that the *rad50S* mutant is deficient for meiotic DSB repair at 34 °C, but partially proficient at 25 °C.

We next asked if the low spore viability and inability to repair meiotic DSBs in *rad50Δ* and *rad50s* was due to a defect in removing covalently bound Rec12^{Spo11} from the

DSB ends. Based on previously published procedures (17, 34), we developed an assay (the DNA-linked protein detection or DLPD assay) to detect the presence of covalently bound Rec12^{Spo11} on the DNA (see Materials and Methods). As shown in Fig. 1d, both *rad50Δ* and *rad50S* show a strong presence of covalently bound Rec12^{Spo11} 6 hours after the initiation of meiosis, whereas in WT cells Rec12^{Spo11} has been removed from the DNA. We consistently find higher covalently bound Rec12 levels in *rad50S* compared to *rad50Δ*, please see discussion for possible explanations. We conclude from these experiments that *rad50S* is a temperature sensitive mutation which, like *rad50Δ*, is defective in Rec12^{Spo11} removal, leading to the inability to repair meiotic DSBs and a strong reduction in spore viability at restrictive temperature.

***rad50S* is a separation of function mutant which is proficient for meiotic recombination functions independent of Rec12^{Spo11} removal.**

To find out if *rad50S* is only deficient for Rec12^{Spo11} removal, or also for other recombination-related functions which are defective in *rad50Δ*, we compared different meiotic phenotypes between *rad50Δ* and *rad50S*.

The repair of meiotic DSBs results in genetically detectable recombination when the homologous chromosome is used as a repair template, while recombination with the sister chromatid is usually silent. We determined meiotic recombinaton levels (in surviving spores) in 3 genetic intervals in *rad50Δ* and *rad50S* mutants at both 25 °C and 34 °C. As shown in Fig. 2A, meiotic intergenic recombination levels in *rad50Δ* are strongly reduced in all intervals tested at both 25 ºC and 34 ºC (a 28 fold reduction on

177 average). Surprisingly, recombination levels in *rad50S* are similar to those of WT cells at
178 both temperatures (1.0 fold on average).

179 The *S. pombe ade6-M26* mutation creates a hotspot for meiotic recombination.
180 This hotspot activity has been associated with the creation of a meiosis-specific
181 micrococcal nuclease (MNase) hypersensitive site through chromatin remodelling (23).
182 To see if this meiosis-specific MNase sensitivity is affected in MRN mutants, we
183 performed an MNase assay on a synchronised meiotic time course. As shown in Fig. 2B,
184 meiosis-specific chromatin remodelling at *ade6-M26* (see black arrow in WT at 3 hrs) is
185 defective both in *rad50Δ* and *rad32*^{mre11}*Δ*, whereas remodelling is proficient in *rad50S*.

186 *S. pombe* LEs are similar to the axial elements of the synaptonemal complex in
187 other eukaryotes, and are believed to play a role in meiotic chromosome organisation and
188 recombination (4). To asses the role of the MRN complex in LE formation, we looked at
189 LEs in meiotic spread preparations of *rad50Δ* and *rad50S* mutants (Fig 3). We found that
190 LEs in *pat1-114* meiosis (used for its high degree of synchrony and relative stability of
191 *rad50Δ* diploids, see discussion; Fig 1a) are shorter and lack the networks, bundles and
192 long elements normally found in *pat1*$^{+}$ meiosis (4). We therefore classified *pat1-114* LEs
193 as 1a (short LEs) and 1b (longer LEs). LEs in *rad50S* are slightly longer and more
194 abundant than in WT (as signified by the increase of class 1b in this mutant compared to
195 WT; Fig 3b), but are totally absent in *rad50Δ*. We quantified the position of the spindle
196 pole body (SPB) relative to the nucleolus (which is associated with the rDNA near both
197 ends of chromosome 3) in *rad50Δ* cells to confirm that these cells went into meiosis. In
198 mitotic cells the SPB is found next to the centromeres, but upon induction of meiosis the

centromeres and telomeres switch position, and the telomeres associate with the SPB (9). As shown in the bottom left panel of Fig. 3b, the majority of the *rad50Δ* cells showed a meiotic SPB configuration, confirming that these cells underwent meiosis.

We conclude from this set of experiments that *rad50S* is a separation of function mutant which is only deficient for Rec12^{Spo11} removal, but proficient for other Rad50-dependent functions related to meiotic DSB repair.

The Rad32^{Mre11} nuclease activity is required for Rec12^{Spo11} removal

Although various observations suggest that the nuclease activity of Mre11 is responsible for Spo11 removal in *S. cerevisiae*, these experiments could not distinguish between a role for Mre11 in Spo11 removal, or the subsequent exonucleolytic resection, and an involvement of the Mre11 nuclease activity in Spo11 removal has not been confirmed experimentally (19). The possibility that a nuclease other than Mre11 could be responsible for Spo11 removal is illustrated by the observation in *S. pombe* that the nuclease activity that degrades the C-rich strand at the telomeres (to create a 3' G-rich strand overhang) is dependent on, but not provided by the MRN complex, and that a second ssDNA specific endonuclease, Dna2 (3), is recruited by MRN and provides the nuclease activity (38, 37).

To distinguish between the possibilities that Rec12^{Spo11} is removed by Rad32^{Mre11}, or by another nuclease recruited and/or controlled by MRN, we created a *rad32^{mre11}-D65N* nuclease dead mutant. This mutant is the equivalent of the well characterised *S. cerevisiae mre11-D56N* mutant, which has been shown to be deficient for nuclease

activity and proficient for MRN complex formation (18). Also in *S. pombe*, the *rad32^{mre11}-D65N* mutant is proficient for MRN complex formation (Nick Rhind, personal communication).

We first studied meiotic spore viability of *rad32^{mre11}-D65N* in combination with *rad50Δ* and *rad50S* mutants (Fig. 4a). The spore viability of *rad32^{mre11}-D65N* and *rad32^{mre11}-D65N rad50S* is strongly reduced compared to *rad50Δ*, whereas in *rad32^{mre11}-D65N rad50Δ* spore viability is rescued approximately to *rad50Δ* levels. We believe that these observations might reflect MRN complex stability in *rad50S* and *rad32^{mre11}-D65N* mutants, versus instability in *rad50Δ*. An intact but nuclease deficient complex might block access of DSB ends to (as yet unknown) alternative removal activities, further decreasing spore viability. The double mutants with *rad50S* (and *rad50Δ*) do not show a lower spore viability than the single *rad32^{mre11}-D65N* mutant, suggesting that they are all defective in the same Rec12^{Spo11} removal pathway.

The extremely low spore viability of *rad32-D65N* precluded a reliable measurement of meiotic recombination. Among 280 survivors, not a single recombinant was detected. However, prolonged snail enzyme digestion (which is used to kill vegetative cells) further reduced the number of survivors (unpublished observation), and it is therefore likely that these survivors do not result from meiotic spores but represent a small fraction of vegetative cells that resist snail enzyme treatment.

Using the DLPD assay to detect covalently bound Rec12^{Spo11} (Fig. 4b), we found that *rad32^{mre11}-D65N* is indeed defective in Rec12^{Spo11} removal, to a degree similar to that of *rad50S*. As in the spore viability assay, the defect is less pronounced in *rad50Δ* and

243 *rad32^{mre11}-D65N rad50Δ*, whereas the defect in *rad32^{mre11}-D65N rad50S* is comparable to
244 that of *rad32^{mre11}-D65N* and *rad50S*. Taken together, these data show that the Rad32^{Mre11}
245 endonuclease activity is required for Rec12^{Spo11} removal in meiosis.
246
247 **Ctp1 is required for Rec12^{Spo11} removal**
248 Sae2 shows only weak homology to Ctp1^{CtIP} (21). *S. cerevisiae sae2* mutants exhibit a
249 *rad50S*-like phenotype and are deficient for Spo11 removal (32, 28). Like *rad50S*, *sae2Δ*
250 is only mildly MMS sensitive (21). In contrast, the sensitivity of *S. pombe ctp1Δ* to
251 ionising radiation (21) and MMS (13) is identical to that of MRN null mutants, and much
252 higher than *rad50S* (13). Using PFGE, it has previously been shown that *ctp1Δ* is
253 deficient for meiotic DSB repair (1). To test functional conservation between Sae2 and
254 Ctp1^{CtIP}, we analysed *ctp1Δ* for its ability to remove covalently bound Rec12^{Spo11} from the
255 DNA. As shown in Fig 5a, *ctp1Δ* is as defective in Rec12^{Spo11} removal as *rad32^{mre11}-*
256 *D65N*, suggesting a functionally conserved role for Ctp1/Sae2 homologues in Spo11
257 removal. Whereas covalently bound Rec12 levels are similar in *ctp1Δ* and a *ctp1Δ rad32-*
258 *D65N* double mutant, these levels are comparatively lower in *rad50Δ* and a *ctp1Δ*
259 *rad50Δ* double mutant.
260 As shown in Fig. 5b, meiotic spore viability in *ctp1Δ* is reduced below *rad50Δ*
261 levels, similar to that of *rad32-D65N.* The spore viability is rescued to *rad50Δ* levels in a
262 *ctp1Δ rad50Δ* double mutant, but not in a *ctp1Δ rad32-D65N* double mutant, possibly
263 reflecting that the MRN complex remains intact in *ctp1Δ*. As explained above for *rad32-*
264 *D65N*, the extremely low spore viability in *ctp1Δ* precludes a reliable measurement of

meiotic recombination.

We also studied meiosis specific chromatin remodelling, and found that in *ctp1Δ* the *ade6-M26* MNase hypersensitive site is present in meiosis (Fig. 5c) and thus that Ctp1 is not required for this chromatin remodelling event.

Discussion

The Rad32^{Mre11} nuclease activity and Ctp1 are required for Rec12^{Spo11} removal

Several studies have suggested that the Mre11 nuclease activity might be responsible for Spo11 removal. Moreau et al. (27) found that a *S. cerevisiae mre11* nuclease dead mutant was deficient in meiotic DSB end resection and proposed that Mre11 is responsible for removing Spo11. However, this study could not distinguish between a role of Mre11 in (endo)nucleolytic Spo11 removal versus a role in (exo)nucleolytic resection downstream of Spo11 removal. Similarly, the presence of the Spo11 removal product (Spo11 with a covalently attached nucleotide) has not been studied in a *mre11* nuclease dead mutant (28). In this study, we thus provide the first direct demonstration that the Rad32^{Mre11} nuclease activity is indeed required for Rec12^{Spo11} removal in meiosis.

We have shown that *rad50Δ*, *rad50S, rad32^{mre11}-D65N* and *ctp1Δ* are defective in removing Rec12^{Spo11} from the DNA. We consistently see higher levels of covalently bound Rec12^{Spo11} in *rad50S*, *rad32^{mre11}-D65N* and *ctp1Δ* compared to *rad50Δ* (e.g. see Fig. 1d, 4b and 5a). This might be (partially) due to a reduced viability of *rad50Δ* (approximately 25 % of *rad50Δ* cells are dead; 12). However, this reduced viability is unlikely to account fully for the 3 fold reduction in meiotic DSB formation in *rad50Δ* (41). Also, levels of covalently bound Rec12^{Spo11} in *ctp1Δ* are higher than in *rad50Δ* and only very slightly reduced compared to *rad50S* and *rad32^{mre11}-D65N*, while the growth defect in *ctp1Δ* is comparable to that of MRN null mutants (21, 1). These observations suggest that Rad50 is required for WT levels of meiotic DSBs. In *S. cerevisiae*, *RAD50* is absolutely required for meiotic DSB formation (6, 16).

The almost identical Rec12^{Spo11} removal defects of *rad50S* and *rad32^{mre11}-D65N* suggests that Rad50 somehow controls the Rad32^{Mre11} nuclease activity. Based on structural studies, it has previously been proposed that ATP driven directional switching of Rad50 controls the Mre11 nuclease activity (14). Interestingly, the *rad50S* mutation is found in a putative protein interaction site, and, based on structural studies, it has previously been proposed that this site might interact with Sae2 (15). A recent study (33) showed that CtIP interacts directly with the MRN complex. As the Rec12^{Spo11} removal defect in *ctp1Δ* is also similar to that of *rad32^{mre11}-D65N*, this opens up the possibility that CtIP/Sae2 controls the Mre11 nuclease activity through its interaction with Rad50.

The most straightforward interpretation of our data is that the Rad32^{Mre11} nuclease is directly responsible for Rec12^{Spo11} removal. However, a recent study (20) showed that purified *S. cerevisiae* Sae2 possesses a nuclease activity which cleaves hairpin DNA structures *in vitro*, cooperatively with the MRN complex (called MRX in *S. cerevisiae*). Purified MRX promotes cleavage by enlarging a single strand gap in the DNA opposite of the Sae2 cleavage site. This raises the possibility that coordinated action of Mre11 and Sae2 nuclease activities might be required, and that Sae2 is ultimately responsible for Spo11 removal.

MRN null mutants are defective for meiosis-specific chromatin remodelling, LE formation and recombination

We find that meiotic recombination in *rad50Δ* is reduced aproximately 28 fold, in line

313 with the previously reported reduction in meiotic recombination in *rad32$^{mre11}\Delta$* (36). This
314 reduction might be partially due to reduced DSB formation in this mutant (see previous
315 section). We showed that in *rad50Δ* and *rad32Δ* mutants *ade6-M26* chromatin
316 remodelling is almost completely abolished. In contrast, in *S. cerevisiae* only Mre11 is
317 required for meiosis-specific chromatin remodelling at meiotic recombination hotspots,
318 whereas Rad50 is dispensable for this process (29). The role of meiosis-specific
319 chromatin remodelling, and the role of MRN therein, is not well understood, but it is
320 probably involved in meiotic DSB formation and/or subsequent recombinational repair.
321 *S. cerevisiae* Mre11 has also been implicated in chromatin remodelling during mitotic
322 DSB repair (39).
323 We found that LE formation is totally abolished in *rad50Δ*. A potential caveat is
324 that these experiments were performed in a *pat1-114* mutant, which shows shortened
325 LEs, while networks, bundles and longer LEs (as found in *pat1$^+$*; 4) are absent. Because
326 of the extreme instability of *h$^+$*/*h$^-$ rad50Δ*/*rad50Δ* diploids (12) we were not able to
327 perform these experiments in *pat1$^+$* meiosis. In a *S. cerevisiae rad50Δ* mutant, shortened
328 axial cores are formed, but they never form a tripartite SC structure (2). Most
329 recombination defective mutants studied so far do form (often aberrant) LEs (24, 25, 26).
330 Another mutant in which LE formation is abolished is *rec10Δ* (26), and it was later
331 shown that Rec10 is an LE component (22). Our observations raise the possibility that
332 Rad50 fulfils a structural role or might regulate an early step in LE formation.
333
334

Rec12^{Spo11} removal is not required for meiosis-specific chromatin remodelling, LE formation and recombination

Whereas *rad50S* is defective in Rec12^{Spo11} removal, we found that it is proficient for meiotic recombination, meiosis-specific chromatin remodelling and LE formation, functions which are all defective in *rad50Δ*.

Meiotic recombination levels and levels of MNase sensitivity at *ade6-M26* are very similar in *rad50S* compared to WT. However, we found that LEs in *rad50S* are elongated compared to WT. We speculate that this might be related to the prolonged presence of meiotic DSBs in *rad50S*, maybe allowing more time for the LEs to mature. In *S. cerevisae rad50S*, like in *rad50Δ*, no fully formed synaptonemal complex is found. However, axial cores in *rad50S* are longer than in *rad50Δ*, and sometimes short stretches of tripartite structure are formed (2).

Fig. 6 shows a diagram which explains our interpretation of the relation between Rec12^{Spo11} removal, meiotic spore viability and meiotic recombination in *rad50Δ* and *rad50S*. Both *rad50Δ* and *rad50S* (at restrictive temperature) are deficient for the removal of covalently bound Rec12^{Spo11} after meiotic DSB formation, leading to low spore viability. However, the presence of viable spores in these mutants suggests that a small fraction of cells is able to remove Rec12^{Spo11} (through an as yet unknown alternative mechanism), allowing repair of the DSB and viable spore formation. In *rad50Δ* these survivors show a strong reduction in recombination rates, suggesting that they survive through a non-recombinogenic survival mechanism (possibly non homologous end joining or recombinational repair using the sister chromatid as a template). The *rad50S*

cells are proficient for meiotic recombination (once Rec12^{Spo11} has been removed) and the surviving spores therefore show normal meiotic recombination levels.

Conclusions and outlook

Although it has been predicted that the *S. cerevisiae* Mre11 nuclease activity is responsible for Spo11 removal, this has not been confirmed experimentally. This study demonstrates for the first time that the Rad32^{Mre11} nuclease activity is required for Rec12^{Spo11} removal. The Rec12^{Spo11} removal defect in *ctp1Δ* suggests functional conservation between the distantly related Sae2 and Ctp1^{CtIP} proteins. We also confirmed that *S. pombe rad50S* is defective in Rec12^{Spo11} removal. Our finding that the temperature sensitive *rad50S* mutant is proficient for meiotic recombination, meiosis-specific chromatin remodelling and LE formation, functions which are all defective in *rad50Δ*, suggests that involvement of MRN in these functions is independent of Rec12^{Spo11} removal.

Conservation of the involvement of the MRN complex and Ctp1^{CtIP} in Rec12^{Spo11} removal, SC/LE formation and meiosis-specific chromatin remodelling in the distantly related yeasts *S. cerevisiae* and *S. pombe* suggests that these MRN functions might be conserved throughout the eukaryotic kingdom. The analysis of MRN and CtIP functions in meiosis of higher eukaryotes has been hampered by inviability of its mutants. Whereas a mouse *Rad50^{R83I}* mutant (equivalent to the *rad50-K81I* allele used in this study) is inviable, *Rad50^{K22M}* (equivalent to the less well characterised *S. cerevisiae* R20M *rad50S* mutant; 2) is viable but shows only mild meiotic phenotypes (5). These observations

might reflect either that the similar, but not identical, amino acid change (K22M in mouse versus R20M in *S. cerevisiae*) might not confer a Spo11 removal defect, or that the MRN complex is not involved in Spo11 removal in mice. Another study (8) suggests that the mouse MRN complex is involved in meiotic prophase progression, chromosome synapsis and recombination.

The findings presented in this study have important implications for our understanding of the role of the MRN complex and $Ctp1^{CtIP}$ in meiotic recombination, defects of which lead to chromosome non-disjunction, infertility and chromosomal abnormalities.

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560 Table 1. *S. pombe* strains used in this study

Strain *	Genotype
265	*h- smt0 leu1-32 ura4-D18*
251	*h+ ura4-D18 leu1-32*
260	*h- smt0 rad50::kanMX6 leu1-32 ura4-D18*
284	*h+ rad50::kanMX6 leu1-32 ura4-D18*
258	*h+ rad50-K81I leu1-32 ura4-D18*
259	*h- smt0 rad50-K81I leu1-32 ura4-D18*
263	*h+ rec12-152::LEU2 leu1-32 ura4-D18*
264	*h- smt0 rec12-152::LEU2 leu1-32 ura4-D18*
261	*h+ rad50::kanMX6 leu1-32 ura4-D18 rec12-152::LEU2*
262	*h- smt0 rad50::kanMX6 leu1-32 ura4-D18 rec12-152::LEU2*
266	*h+ leu1-32 ura4-D18 rec12-152::LEU2 rad50-K81I*
267	*h- smt0 leu1-32 ura4-D18 rec12-152::LEU2 rad50-K81I*
805	*h- smt0 ura4-D18 rad32-D65N*
814	*h+ rad32-D65N ura4-D18*
810	*h- smt0 ura4-D18 rad32-D65N rad50::kan*
811	*h+ ura4-D18 rad32-D65N rad50::kan*
812	*h- smt0 ura4-D18 rad32-D65N rad50S*
813	*h+ ura4-D18 rad32-D65N rad50S*
352/354	*h-/h+ ade6-M216/ade6-M210 ura4-aim/ura4-aim ura4-D18/ura4-D18 rad50-K81I/rad50-K81I*
611	*h- smt0 ade6-M26 pat1-114 rec12-6HA:kanMX6* †
617	*h- smt0 pat1-114 ade6-M26 rec12-6HA:kanMX6 rad50-K81I*
649	*h- smt0 ade6-M26 rec12-6HA:kanMX6 rad50::kanMX6 pat1-114*
418	*h+ mat1PD17::LEU2 leu1-32 arg6-1*
417	*h- smt0 ade7-152*
411	*h+ mat1PD17::LEU2 leu1-32 rad50-K81I arg6-1*
412	*h- smt0 ade7-152 rad50-K81I*
407	*h+ mat1P::LEU2 leu1-32 rad50::kanMX6 arg6-1*
408	*h- smt0 rad50::kanMX6 ade7-152*
421	*h- smt0 lys7-1 leu1-32*
422	*h+ mat1PD17::LEU2 leu1-32 ade2-17 ura2-10*
405	*h- smt0 lys7-1 leu1-32 rad50::kanMX6*
406	*h+ mat1PD17::LEU2 ade2-17 ura2-10 leu1-32 rad50::kanMX6*
415	*h- smt0 rad50-K81I lys7-1 leu1-32*
416	*h+ mat1PD17::LEU2 rad50-K81I leu1-32 ade2-17 ura2-10*
157	*h- smt0 ade6-M26 pat1-114*
	h+ ade6-M26 pat1-114 leu1-32
	h+ ade6-M26 rad50-kanMX6 pat1-114 leu1-32
	h+ ade6-M26 rad32::ura4 pat1-114 leu1-32
	h+ ade6-M26 rad50-K81I pat1-114 leu1-32
207/209	*h-/h- smt0/smt0 pat1-114/pat1-114 ade6-M210/ade6-M216 ura4-aim/ura4-aim ura4-D18/ura4-D18*
203/205	*h-/h- smt0/smt0 pat1-114/pat1-114 ade6-M210/ade6-M210 ura4-aim/ura4-aim ura4-D18/ura4-D18 rad50::kanMX6/rad50::kanMX6*
148/152	*h-/h- pat1-114/pat1-114 ade6-M210/ade6-M216 ura4-aim/ura4-aim ura4-D18/ura4-D18 rad50-K81I rad50-K81I*
808	*h- smt0 pat1-114 rad32-D65N rec12-6HA:kan ade6-M26*
834	*h- smt0 pat1-114 rad32-D65N rec12-6HA:kan ade6-M26 rad50::kan*
832	*h- smt0 pat1-114 rad32-D65N rec12-6HA:kan ade6-M26 rad50S*
824	*h- smt0 ctp1::kan pat1-114 rec12-6HA:kan*

561

562 *Numbers are from the strain collection of EH. † *rec12-6HA-kanMX* was created in the

563 lab of Dr. Ohta. Other unpublished strains/constructs for this study were created by EH.

Figure Legends

Figure 1

The *rad50S* mutation is temperature sensitive for meiotic spore viability and defective for meiotic DSB repair and Rec12^{Spo11} removal. a) Meiotic spore viability relative to WT in different strains at 25 ºC and 34 ºC. Error bars show standard deviation, values are the average of 3 independent experiments. b) Pulsed Field Gel Electrophoresis of a synchronised meiotic *pat1*$^{+}$ *rad50S* culture at 25 ºC and 34 ºC. The bands labelled "I/II/III" correspond to the intact chromosomes, the smears labelled "DSB" correspond to broken DNA fragments. c) Meiotic progression of the timecourse presented in Fig. 1b is expressed as the number of cells that have completed meiosis I at different time points. d) Slot blot showing the presence of covalently bound Rec12^{Spo11} on the DNA 6 hours after meiotic induction at 34 °C. At time point 0 no Rec12^{Spo11} signals are visible (data not shown). The arrow indicates where the top and bottom fractions of the CsCl gradient have been loaded. The bulk of the DNA is found in fractions 5, 6 and 7, which show the strongest Rec12^{Spo11} signal in *rad50* mutants.

Figure 2

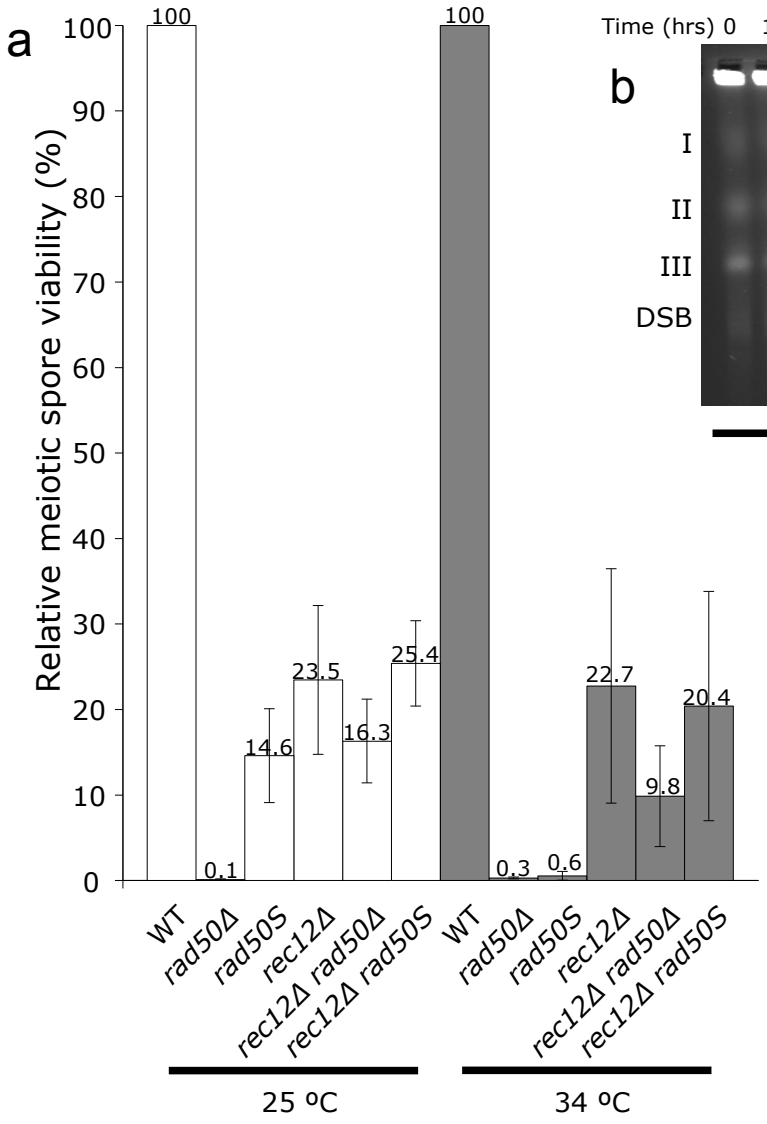
The *rad50S* mutant is proficient for meiotic recombination and meiosis-specific nucleosome remodelling, both functions are defective in *rad50Δ*. a) Meiotic intergenic recombination levels (in surviving spores) in *rad50Δ* are strongly reduced in different genetic intervals at both 25 ºC and 34 ºC. However, *rad50S* is proficient for

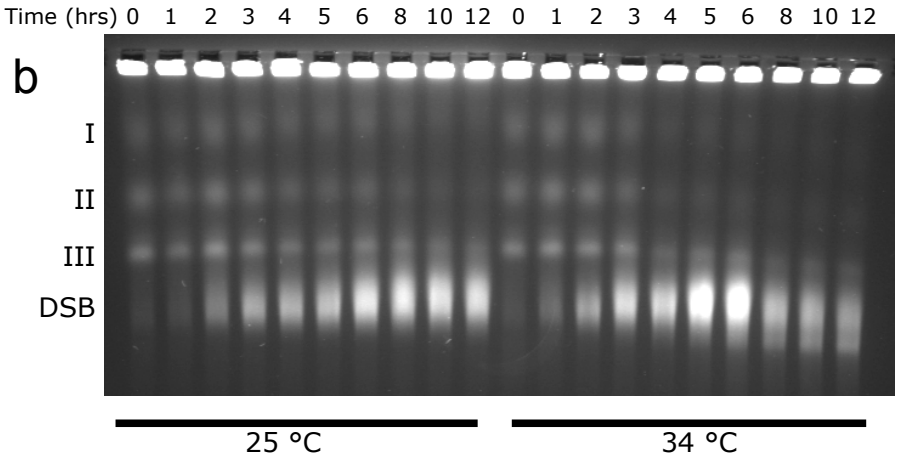
586 recombination at both temperatures. b) Meiosis-specific nucleosome remodeling at *ade6-*
587 *M26* (see black arrow in WT at 3 hrs) is defective at 34 °C in *rad50Δ* (and *rad32*^{mre11}*Δ*),
588 whereas *rad50S* is proficient.
589
590 **Figure 3**
591 The *rad50S* mutation is proficient for linear element formation, whereas linear elements
592 are absent in *rad50Δ*. a) Electron micrographs of lysed and spread meiotic nuclei. Linear
593 elements in *pat1-114* meiosis (used for its high degree of synchrony) are shorter than in
594 WT (*pat1*$^{+}$; 4) whereas networks and bundles are not detected. Linear elements in
595 *pat1-114* meiosis were classified as 1a (short linear elements) and 1b (longer linear
596 elements). Linear elements in *rad50S* are slightly longer and more abundant than in WT.
597 Linear elements are absent in *rad50Δ*. Bottom two panels (*rad50Δ*) illustrate typical
598 spindle pole body (SPB) orientation in mitotic (opposite of nucleolus; NLL) and meiotic
599 cells (next to nucleolus). This allows the distinction between mitotic and meiotic cells
600 and confirms that *rad50Δ* is undergoing meiosis in the absence of linear elements. b) Left
601 top and middle panels: Quantification of the linear element classes 1a and 1b at different
602 time points. Class 1b is more abundant in *rad50S* compared to WT. Bottom panel, left:
603 Quantification of *rad50Δ* cells (without linear elements) containing a spindle pole body
604 configuration indicative of meiosis. At later time points cells start to form ascus and
605 spore walls, making the cells resistant to lysis which leads to an artifactual under-
606 representation of meiotic cells. Right panels: Quantification of DAPI visualised elongated
607 (horse tail) nuclei indicative of meiotic prophase. The percentage of cells with more than

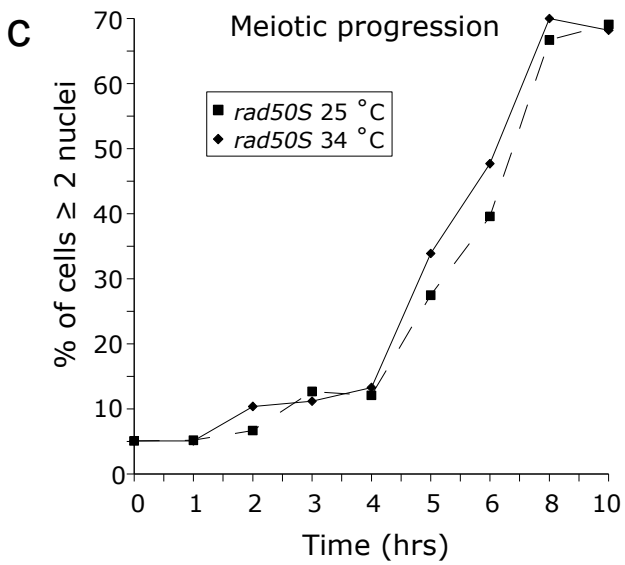
608 one nucleus indicates progression through the first and second meiotic divisions. All
609 quantifications are based on at least 100 cells per timepoint.
610
611 **Figure 4**
612 The *rad32^{mre11}-D65N* mutation is defective for Rec12^{Spo11} removal. a) Analysis of spore
613 viability epistasis between *rad50Δ*/*rad50S* and *rad32^{mre11}-D65N*. Note that the graph only
614 shows the lower range (lower than 0.15 %) of the spore viability. Error bars show
615 standard deviation, values are the average of three independent experiments. b) Analysis
616 of Rec12^{Spo11} removal in *rad50Δ*/*rad50S* and *rad32^{mre11}-D65N* at 34 °C. Levels of
617 covalently bound Rec12^{Spo11} are increased in *rad32^{mre11}-D65N* mutant strains. The arrow
618 indicates where the top and bottom fractions of the CsCl gradient have been loaded. The
619 bulk of the DNA is found in fractions 5, 6 and 7, which show the strongest Rec12^{Spo11}
620 signal in *rad50* mutants.
621
622 **Figure 5**
623 a) A *ctp1* deletion is deficient in removing Rec12^{Spo11} from the DNA in meiotic cells. The
624 defect is comparable to that of *rad50S* and *rad32^{mre11}-D65N* strains. b) Meiotic spore
625 viability is strongly reduced in *ctp1Δ*, similar to that of *rad32-D65N*. c) *ctp1Δ* is
626 proficient for *ade6-M26* chromatin remodelling (see black arrow).
627
628 **Figure 6**
629 Interpretation of the observed meiotic phenotypes in *rad50Δ* and *rad50S*. Both *rad50Δ*

630 and *rad50S* (at restrictive temperature) are deficient for the removal of covalently bound
631 Rec12^{Spo11} after meiotic DSB formation, leading to low spore viability. However, a small
632 fraction of cells is able to remove Rec12^{Spo11} (through an unknown mechanism), allowing
633 repair of the DSB and viable spore formation. In the *rad50Δ* mutant these survivors show
634 a strong reduction in recombination rates, suggesting that they survive through a non-
635 recombinogenic survival mechanism. The *rad50S* cells are proficient for meiotic
636 recombination (once Rec12^{Spo11} has been removed) and the surviving spores therefore
637 show normal meiotic recombination levels.

Figure 1 Hartsuiker et al.







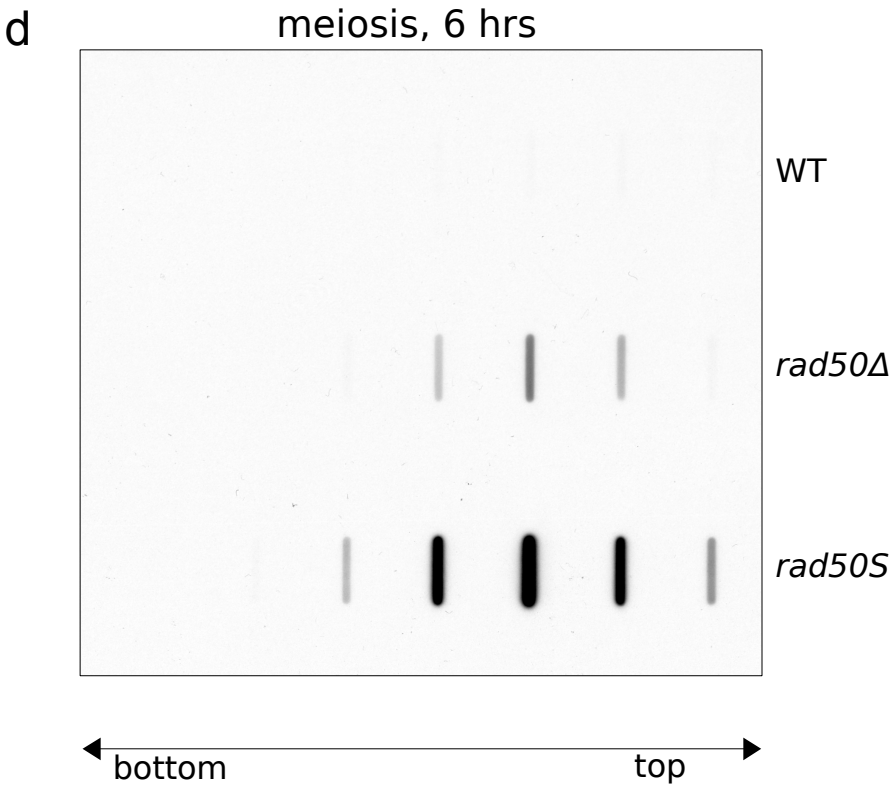
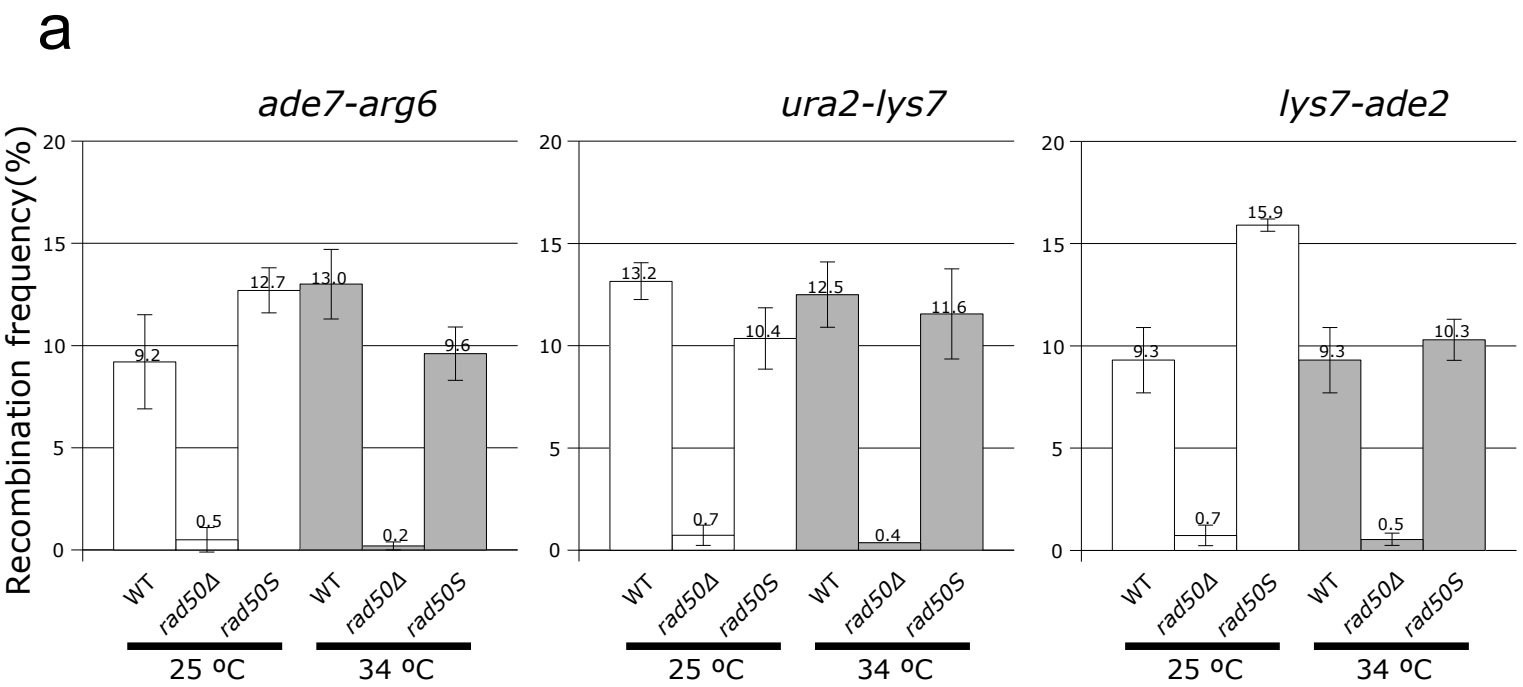


Figure 2 Hartsuiker et al.



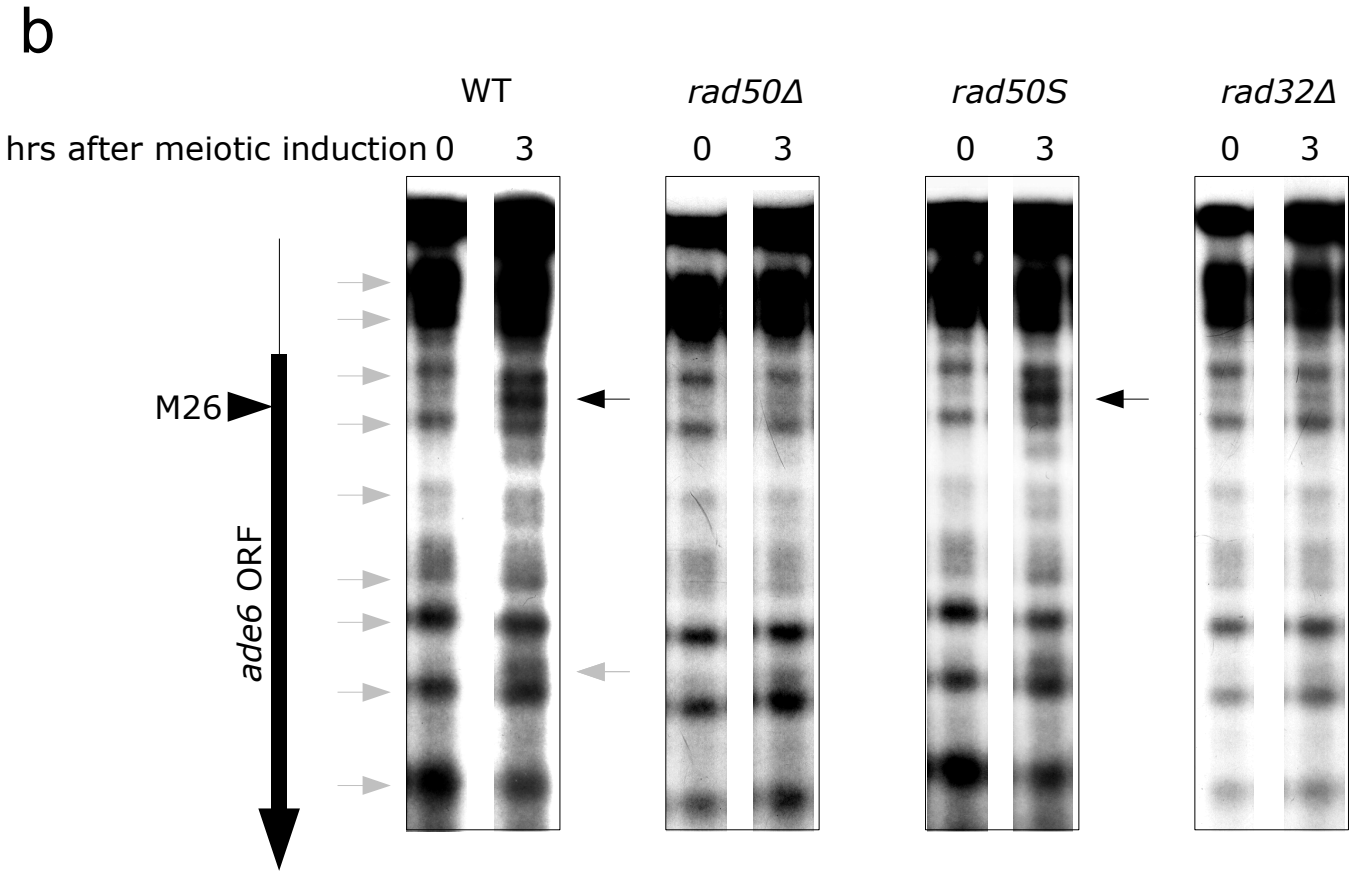
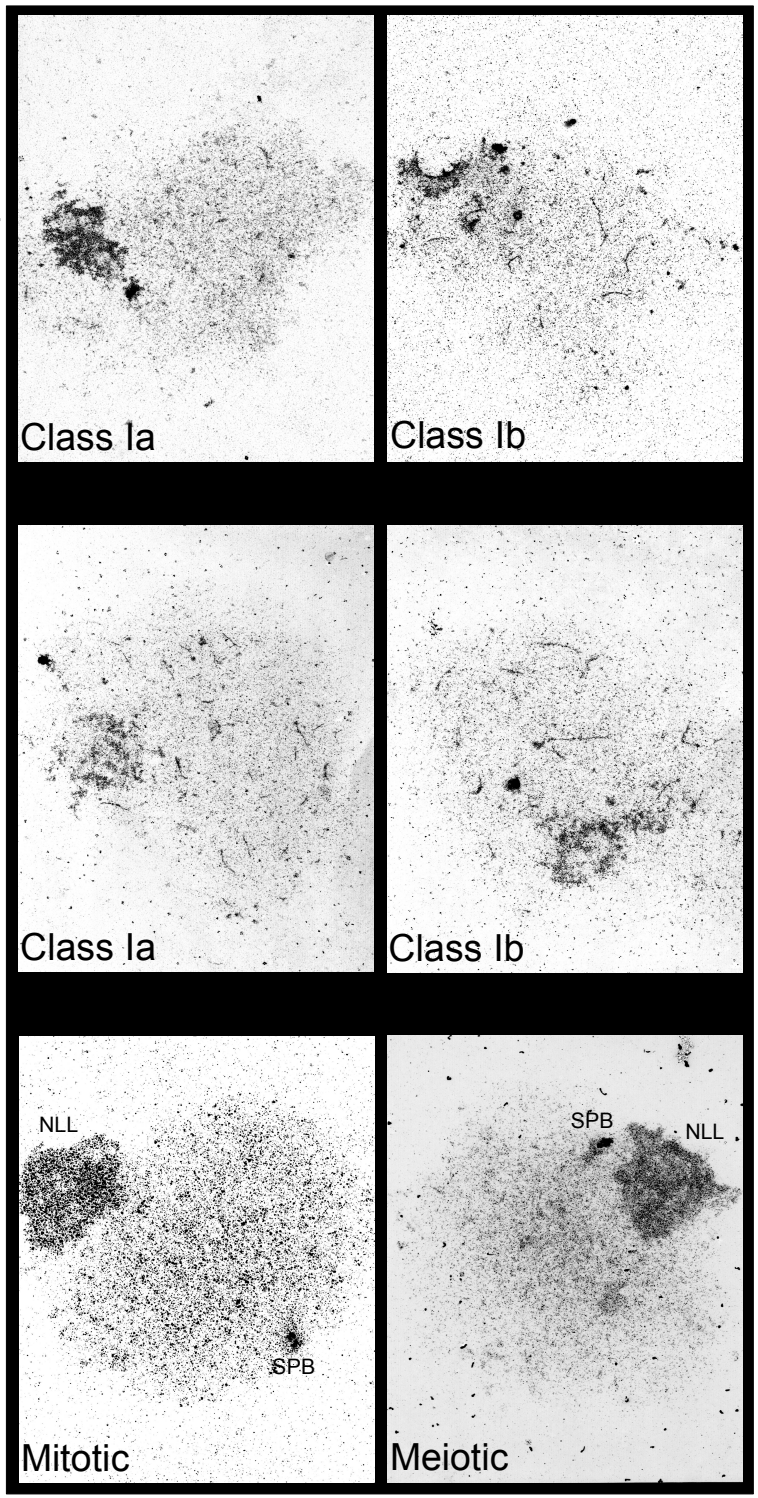


Figure 3 Hartsuiker et al.



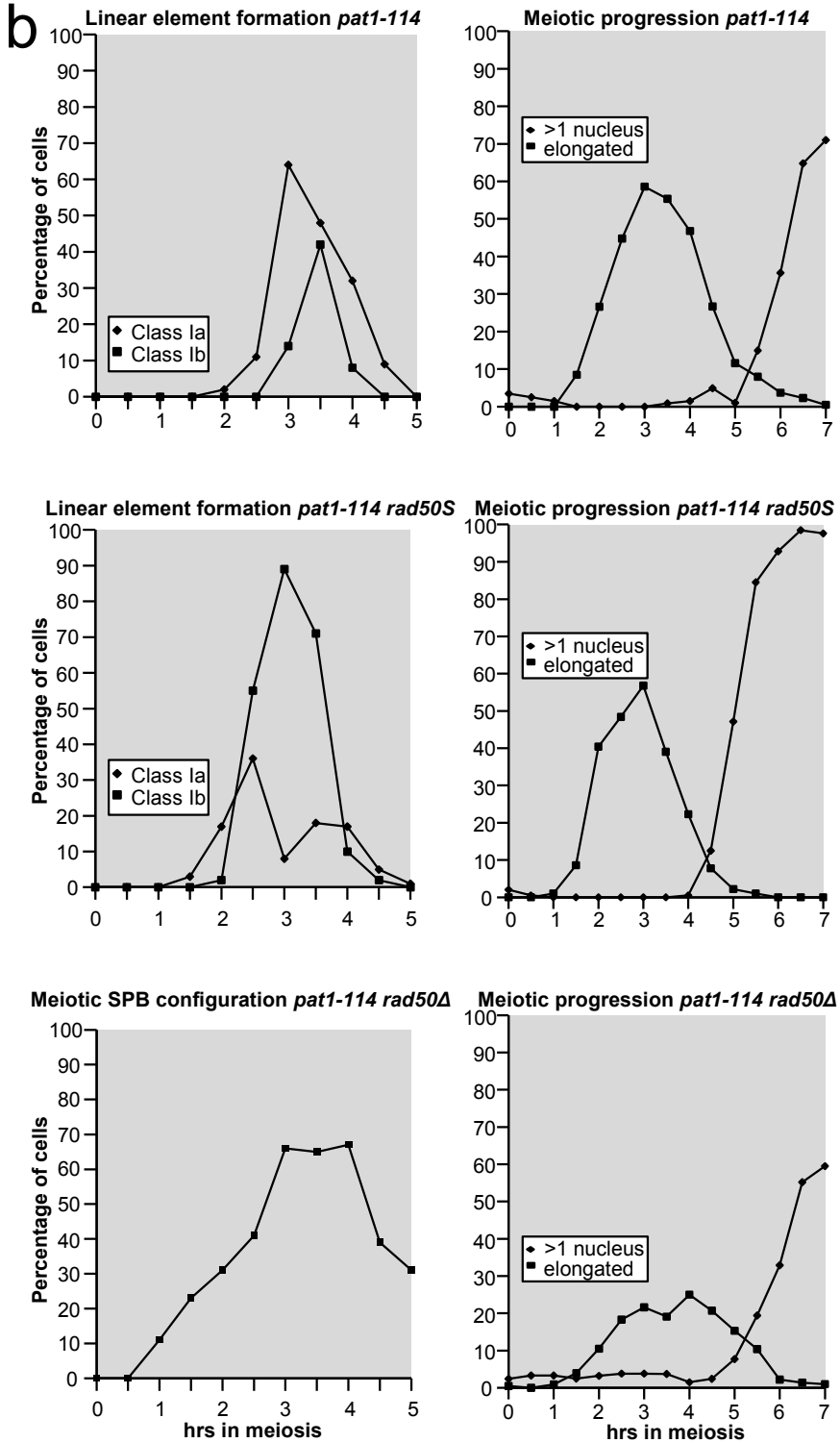


Figure 4 Hartsuiker et al.

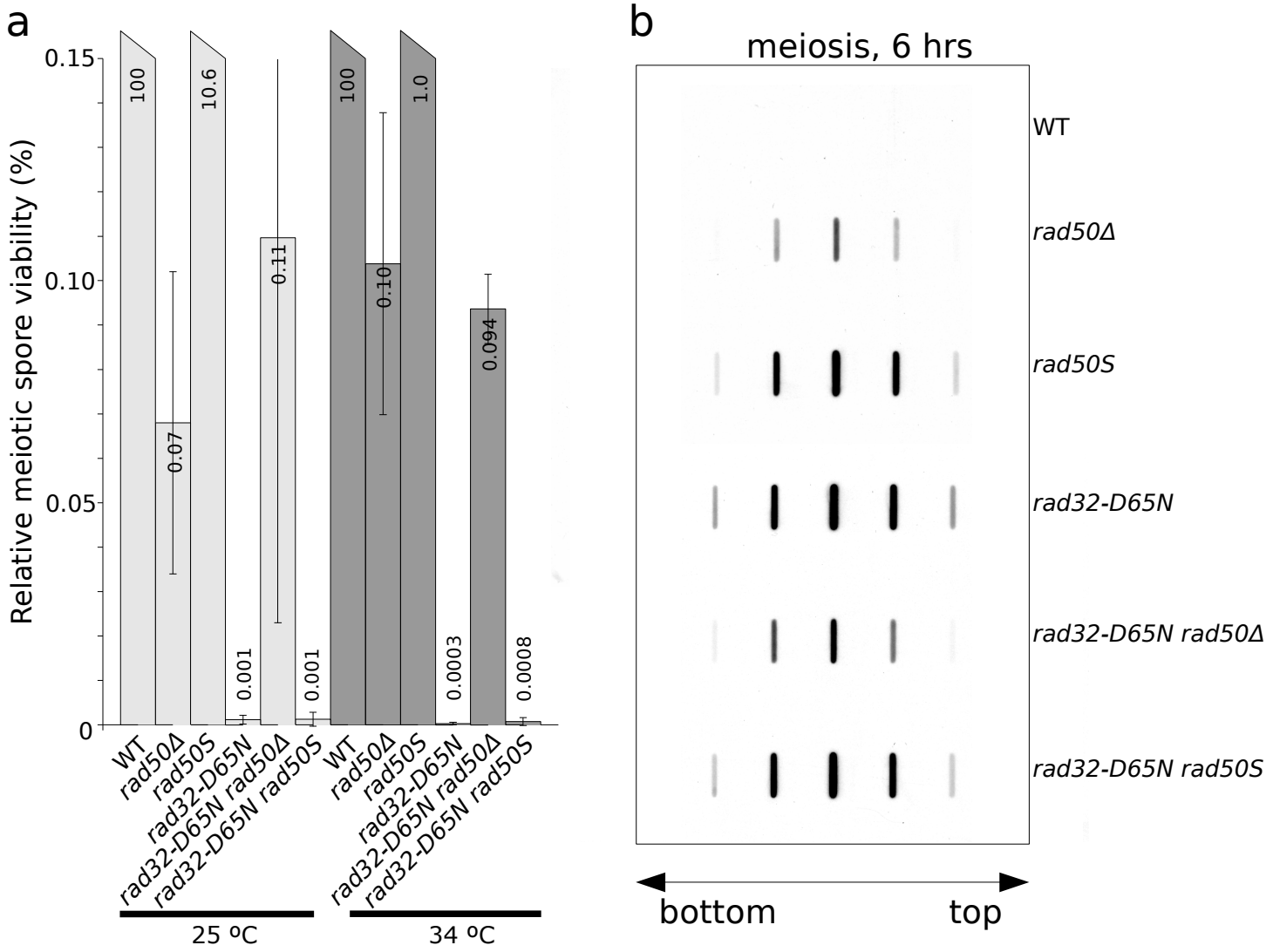


Figure 5 Hartsuiker et al.

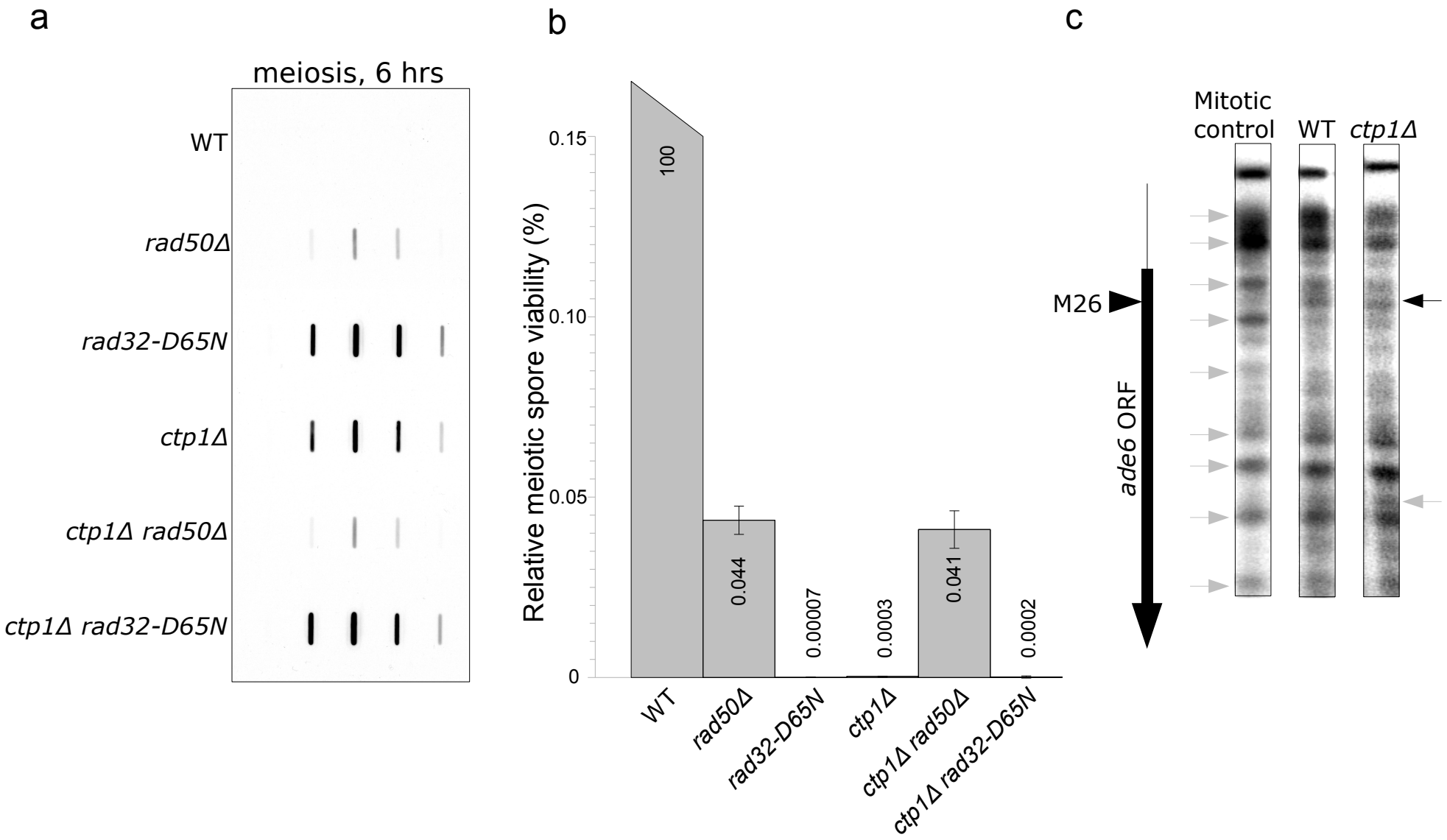


Figure 6, Hartsuiker et al.